Research article

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Use of spent brewer's yeast in L-(+) lactic acid fermentation

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The application of by-products from the brewing industry in lactic acid (LA) production was investigated in order to replace expensive nitrogen sources (such as yeast extract) with cheaper and renewable nitrogenous materials such as brewer's yeast (BY). In this study, brewer's spent grain (BSG) hydrolysate was used for L-(+)-LA fermentation by *Lactobacillus rhamnosus* ATCC 7469. The effect of pH control during the fermentation and the addition of various BY contents (5–50 g/L) in BSG hydrolysate on fermentation parameters was evaluated. BY addition significantly increased free amino nitrogen (FAN) concentration (by 25.2% at 5 g/L to 616% at 50 g/L). A strong positive correlation between FAN concentration in the hydrolysate and concentration of L-(+)-LA produced was observed (correlation coefficient of 0.913). A high cell viability of *L. rhamnosus* ATCC 7469 (1.95–3.32 × 10^9 CFU/mL at the end of fermentation) was achieved in all fermentations with the addition of brewer's yeast. The addition of BY increased L-(+)-lactic acid yield and volumetric productivity up to 8.4% (5 g/L) and 48.3% (50 g/L). The highest L-(+)-LA yield (89%) and volumetric productivity (0.89 g/L h^{-1}) were achieved in fermentation of BSG hydrolysate with 50 g/L of BY.

Keywords: lactic acid fermentation; brewer's spent grain; brewer's yeast; free amino nitrogen

Introduction

The major by-products/co-products of the brewing industry are yeast and brewer's spent grain (BSG) *(1)*. Brewer's yeast is relatively inexpensive and is utilised in the production of extracts for fermentation and food industries *(2)*. Yeast cells contain protein, lipid, RNA, vitamins and minerals and are generally recognised as safe (GRAS) *(3)*. Brewer's spent grain consists of solids remaining after wort separation. Approximately 20 kg of BSG (wet basis) is generated for every 100 L of beer produced. About 30 million tons of BSG is produced annually worldwide and is used as animal feed or sent to landfill *(4,5)*.

BSG contains significant amounts of dietary fibre, protein and essential amino acids, as well as modest amounts of lipids, polyphenols, minerals and vitamins. In recent years there has been a significant interest in BSG owing to its chemical composition that allows its re-use (raw material for microbiological or chemical conversion, pharmaceutical, food ingredient, feed, cosmetic or other industries) *(6)*.

Lactic acid has diverse applications in the pharmaceutical, food, textile, leather and chemical industries *(7)*. It recent years, lactic acide (LA) has become an important raw material for the production of biopolymers such as polylactide and chemicals such as 2,3-pentanedione, acetaldehyde and acrylic acid *(8)*. Lactic acid can be produced via two routes, chemical synthesis and microbial fermentation. The racemic mixture of DL-LA is consistently produced by chemical synthesis from petroleum resources, while optically active L-(+)- or D-(−)-LA can be produced by fermentative route from renewable resources *(9)*.

Application of lactic acid in food and food related industries accounts for ~85% of total LA produced *(10)*. The estimated yearly growth of LA demand is 5–8%, with expected annual worldwide LA production of 367,300 metric tons by 2017 *(11)*. However, the high cost of synthetic medium and nitrogen source supplementation, with competition for starch substrates with food application, reduces the economic feasibility of LA production. Therefore, agro and food industry by-products and waste products are considered as suitable substrates for bio-based LA production *(12)*. Recently, because of its renewable nature, lignocellulosic biomass has gained importance with increasing research *(13)*. The process of cellulose bioconversion to LA is carried out in two steps: hydrolysis of cellulosic material to glucose by chemical, physical or mostly enzymatic methods and LA fermentation using the hydrolysate *(14)*.

Lactic acid bacteria (LAB) are a diverse group of Gram-positive bacteria that are found in meat, dairy products and plants which are able to produce LA with high yield and productivity. Some LAB strains are commercially used, such as *Lactobacillus* strains, owing to their tolerance to acidic environments and their ability to be genetically modified for the production of optically pure L-(+)- or D-(−)-LA *(15)*. Lactic acid bacteria require, in addition to a carbon source, nitrogen containing nutrients (peptides, amino acids) for growth and LA production and accordingly the fermentation medium needs to be supplemented with complex protein hydrolysates (peptone, yeast extract, etc.). Commercial protein extracts are expensive and their replacement by low-cost nitrogen-

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containing extracts is required when considering large scale production *(16)*. With regard to the economics of LA fermentation, yeast extract accounts for about 38% of the cost of the fermentation medium. Therefore, there is an increasing interest in the utilisation of cheap renewable nitrogenous substrates such as industrial and agricultural by-products as substitutes for yeast extract in LA fermentation *(17)*.

In this study, spent grain hydrolysates were used for LA fermentation by *Lactobacillus rhamnosus*. The aim of this study was to evaluate pH control and the effect of addition of various brewer's yeast (BY) contents (5–50 g/L) in BSG hydrolysate on fermentation parameters such as LA concentration, its productivity and yield, utilisation of reducing sugar and viability of *L. rhamnosus* cells.

Material and methods

Brewer's spent grain hydrolysate preparation

Brewer's spent grain obtained from the production of lager was dried at 40°C for 12 h. Dried BSG was finely ground in a DLFU mill from Bühler-Miag (Braunschweig, Germany). Prior to the fermentation the BSG hydrolysis was optimised. Enzyme dosage was according to the manufacturer's recommendation but was subsequently increased according to the results of this work. All commercial enzymes used in BSG hydrolysis (Termamyl SC, SAN Super 240 L, and Celluclast 1.5 L®) were provided by Novozymes (A/S Bagsvaerd, Denmark). Each enzyme (Termamyl SC®, SAN Super 240 L® and Celluclast 1.5 L®) was assessed alone and then a combination of the three enzymes was used according to the best results for each enzyme.

For hydrolysate production, 50 g of dry BSG was mixed with 300 mL of distilled water and the pH adjusted to 5.5 with the addition of 10% phosphoric acid (H_3PO_4) prior to hydrolysis. BSG hydrolysis was carried out as previously described *(18)* using an automated mashing water bath (Glasbläserei, Institut für Gärungs Gewerbe, Berlin) by sequentially adding the following enzymes: 0.3 mL Termamyl SC (1 h at 90°C), 0.3 mL SAN Super 240 L (1 h at 55°C) and 5.0 mL Celluclast 1.5 L (10 h at 45°C) at 180 rpm. Prior to the addition of Celluclast 1.5 L, the pH was adjusted to 5 with the addition of 10% H_3PO_4. After enzymatic hydrolysis, the BSG hydrolysate was cooled to 20°C and centrifuged at 2563***g*** for 20 min. The hydrolysate was separated from solid residue and used in subsequent fermentations. The pH was adjusted to 6.5 with the addition of 1 M NaOH.

Spent yeast was obtained from a lager fermentation at AD BIP-Belgrade Beer Industry and was debittered (by water) and dried at high temperature. Dry brewer's yeast was added to the BSG hydrolysate in the form of dried inactivated product before autoclaving. During autoclaving, the yeast cells were degraded (Figure 1). The BY contents in the hydrolysate were 5, 10, 20, 30, 40 and 50 g/L with the addition of the corresponding content of dry BY. After this, liquid hydrolysate was sterilised at 121°C for 15 min and used as a fermentation medium. The dry yeast and spent grain composition are given in Table 1.

BSG hydrolysate contained 25 g/L of reducing sugar. With the addition of BY to the hydrolysate, the reducing sugar concentration increased (Table 2). By increasing the BY content to 50 g/L, the reducing sugar concentration increased to 44 g/L. To avoid the different reducing sugar concentrations in the BSG hydrolysate affecting fermentation performance, the reducing sugar concentration was controlled at 50 g/L by the addition of a sterile 70% (w/v) glucose solution.

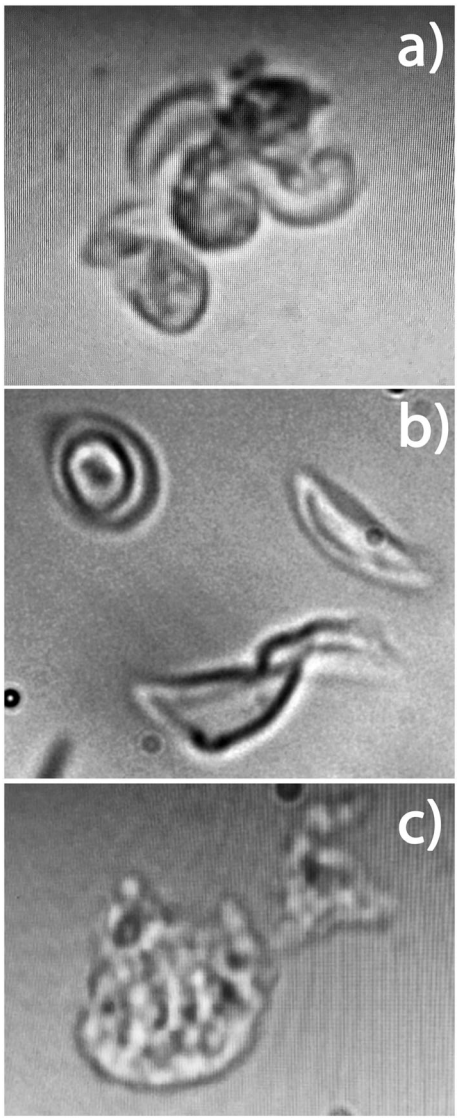

Figure 1. Brewer's yeast cell cytology during autoclaving.

Table 1. Brewer's yeast (BY) and brewer's spent grain (BSG) composition[a]

Parameter (%)	BY	BSG
Protein	45.88 ± 0.78	26.37 ± 0.34
Lipid	2.87 ± 0.13	8.18 ± 0.26
Moisture	6.44 ± 0.04	4.56 ± 0.07
Cellulose	2.57 ± 0.07	15.28 ± 0.44
Ash	8.16 ± 0.31	4.21 ± 0.17

[a]Values represent means ± standard deviation calculated from three determinations.

Microorganisms

Lactobacillus rhamnosus ATCC 7469, a homofermentative strain, was obtained from the American Type Culture Collection (ATCC, Rockville, MD. USA). A stock culture of *L. rhamnosus* was activated as previously described *(19)*. The inoculum was prepared by

Table 2. Free amino nitrogen (FAN), total nitrogen and reducing sugar in BSG hydrolysate with the addition of BY[a]

brewer's yeast (g/L) in BSG hydrolysate	FAN (mg/L)	Total nitrogen (g/L)	Reducing sugar (g/L)
0	55.0 ± 0.2	0.80 ± 0.01	25.2 ± 0.2
5	68.9 ± 0.2	0.93 ± 0.04	25.9 ± 0.2
10	147.5 ± 1.0	1.75 ± 0.06	26.9 ± 0.2
20	249.2 ± 1.2	2.62 ± 0.06	31.6 ± 0.2
30	296.4 ± 2.0	3.40 ± 0.08	35.3 ± 0.3
40	376.9 ± 2.1	3.96 ± 0.09	39.9 ± 0.2
50	393.61 ± 2.2	4.65 ± 0.10	44.0 ± 0.2

[a]Values represent mean ± standard deviation from three determinations.

transferring 3 mL of the activated culture to 60 mL of MRS (De Man, Rogosa and Sharpe) broth (HiMedia Laboratories Ltd, Mumbai, India). To achieve an appropriate LAB cell count (1×10^9 CFU/mL) the inoculum was incubated statically for 24 h at 37°C.

LA fermentation

All fermentations were performed as batch cultures with shaking (150 rpm, Biosan shaking bath model ES-20, Biosan Ltd, Letonia). The fermentations were performed in 300 mL Erlenmeyer flasks with 200 mL of BSG hydrolysate. The fermentation was started by the addition of inoculum (5% v/v) and held at 37°C. The pH was maintained at 6.2 by the addition of a sterile 30% (w/v) sodium hydroxide solution at 4 h intervals *(19)*.

Analytical methods

Reducing sugar concentration, calculated as glucose, was determined by the 3,5-dinitrosalicylic acid method *(20)* with a calibration curve at 570 nm using standard glucose solutions. L-(+)-Lactic acid concentration was determined using an enzymatic method [L-(+)-LA assay; Megazyme, Wicklow, Ireland]. Prior to the LA determination, proteins were removed from samples by precipitation according to the procedure detailed in the enzymatic method. The number of viable *L. rhamnosus* cells was determined using the pour plate method. Microaerophilic conditions were maintained during incubation in Petri plates using a double MRS medium layer. Samples were incubated for 48 h at 37°C. Total viable cell number was expressed as log CFU/mL. BSG, BY and BSG hydrolysate (without and with BY) were analysed. The following methods were used for analysis: dry matter content was determined by a standard drying method in an oven at 105°C to constant mass *(21)*; protein content was determined by Kjeldahl method as the total nitrogen and multiplied by factor *(22)* and ash content was determined by slow combustion method at 650°C for 2 h *(21)*. The cellulose content of BSG and BY was determined by a previous method with intermediate filtration *(23)*. The lipid content in BSG and BY was determined by hexane extract method *(24)*. The free amino nitrogen (FAN) concentration in BSG hydrolysate (before and after addition of BY) and BY (before and after autoclaving) was determined by the ninhydrin method *(25)* and reported as mg/L. All chemicals used in experiments were of analytical and microbiological grade.

Statistical analysis

The experiments were performed in triplicate. All values are expressed as means ± standard deviation. Pearson's correlation coefficient was determined for correlation of FAN and LA concentration produced for $p < 0.01$. Mean values of LA concentration, LA yield, volumetric productivity and *L. rhamnosus* cell number were compared by the analysis of variance (one-way ANOVA) followed by Duncan test for mean differences testing (SPSS Statistica 20, IBM Corporation, Armonk, NY, USA). Differences were considered significant at $p < 0.05$.

Results and discussion

The chemical composition of BSG and BY is presented in Table 1. BSG had higher dry matter, lipid and cellulose content than BY, while yeast had significantly higher protein (1.7-fold) and ash (1.9-fold) content than spent grain. FAN, total nitrogen and reducing sugar concentration in BSG hydrolysate increased with yeast addition (Table 2). BY addition significantly increased FAN (by 25% at 5 g/L to 616% at 50 g/L of BY) and total nitrogen concentration (by 17% at 5 g/L to 418% at 50 g/L of BY). Also, the addition of 10–50 g/L BY increased the reducing sugar concentration by 6.5–74.4%. As these results suggest, BY was confirmed as a good source of FAN, minerals and carbon. As recommended, an estimate of brewer's yeast autolysis after autoclaving was performed by determination of FAN and nitrogen concentration in the yeast saline water solution and the results are presented in Table 3. FAN concentration increased by 1.9- to 5.8-fold after autoclaving.

Table 3. Effect of autoclaving on FAN and total nitrogen in BY saline solution[a]

Brewer's yeast (g/L) in saline water	FAN before autoclaving (mg/L)	FAN after autoclaving (mg/L)	BY total nitrogen before autoclaving (g/L)	BY total nitrogen after autoclaving (g/L)
5	29.9 ± 0.3	57.5 ± 0.6	0.14 ± 0.01	0.14 ± 0.01
10	36.7 ± 0.3	100.4 ± 1.4	0.95 ± 0.09	0.95 ± 0.09
20	48.2 ± 0.4	162.2 ± 1.7	1.82 ± 0.13	1.81 ± 0.13
30	60.2 ± 0.4	225.3 ± 2.1	2.61 ± 0.11	2.58 ± 0.11
40	66.7 ± 0.5	388.4 ± 2.4	3.16 ± 0.10	3.18 ± 0.10
50	75.6 ± 0.5	423.2 ± 3.2	3.85 ± 0.10	3.89 ± 0.10

[a]Values represent means ± standard deviation calculated from three determinations.

pH profiles of fermentation of BSG hydrolysates with BY

The pH values in fermentation of BSG hydrolysate, without and with pH control, and with BY addition are reported in Figure 2a. In the fermentation without pH control, the pH decreased quickly (in 8 h of fermentation), which affected the fermentation parameters. With pH control, the pH was closer to the optimum for LAB metabolism (5.5–6.5) *(26)*. As shown in Fig. 2a the addition of BY intensified the pH decrease.

Effect of pH control and BY addition on fermentation

The effect of pH control and addition of BY on fermentation parameters such as LA concentration, productivity and yield, reducing sugar utilisation and *L. rhamnosus* cell viability was assessed. LA concentration in fermentations of BSG hydrolysate, without and with pH control and with BY addition, is shown in Figure 2b. In fermentations without pH control, a low LA concentration was obtained (7.7 g/L) but with pH control the LA concentration increased by 164% (20.4 g/L). Mussatto *et al. (27)* reported a lower LA concentration (12.8 g/L) in the fermentation of BSG hydrolysate by *Lactobacillus delbrueckii* UFV H2B20 (with initial glucose concentration of 50 g/L and with pH control).

With LA fermentations, pH control is crucial as the free LA, even at low concentrations, inhibits both cell growth and further LA formation *(28)*. LA concentration increased significantly (by 15.1% at 5 g/L of BY to 32.8% at 50 g/L of BY) with yeast addition and pH control. The highest LA concentration achieved was 27 g/L, which although insufficient for commercial LA production, can be utilised for in-house wort acidification during beer production.

The reducing sugar concentration in the fermentation of BSG hydrolysate – without and with pH control – together with BY addition is given in Figure 2c. The pH control clearly affected reducing sugar utilisation, with slower utilisation of reducing sugar and conversion to LA in the fermentation without pH control compared with that with pH control (Fig. 2a and b). With pH control, reducing sugar utilisation increased by 118% and with BY addition it improved further. With the BY addition, reducing sugar utilisation increased markedly (by 11.5% at 5 g/L to 22.5% at 50 g/L of BY) compared with the fermentation without the addition. Rakin *et al. (29)* also observed better sugar utilisation in the fermentation of vegetable juice by *L. plantarum* and *L. acidophilus* with the addition of BY.

The cell viability *of L. rhamnosus* in fermentations of BSG hydrolysate, with and without pH control, together with BY addition is presented in Figure 2d. During the fermentation with pH control, *L. rhamnosus* cell viability was higher (by 3%) compared with that without the pH control. High *L. rhamnosus* cell viability ($1.95–3.32 \times 10^9$ log CFU/mL at the end of fermentation) was achieved in all fermentations with BY addition. *L. rhamnosus* cell viability was also higher in fermentations with the addition of 10–50 g/L of BY (by 1.4 at 10 g/L to 3% at 50 g/L of BY) compared with the fermentation without the addition and with pH control. Rakin *et al. (30)* investigated the effect of yeast autolysate on LA fermentation of vegetable juice by *L. acidophilus* NCDO1748. The addition of BY had a positive effect on LA production and led to an increase of *L. acidophilus* viability (by 16.9–27.8%) at the end of fermentation. These results are in agreement with the results reported here regarding the positive effect of yeast addition on lactic acid production and bacterial viability.

The LA concentration correlated with the corresponding FAN concentrations in BSG hydrolysate (correlation coefficient 0.913). A strong positive correlation indicates that FAN had significant and positive effect on LA fermentation and increase of lactic acid concentration.

LA yield and volumetric productivity in the fermentation of BSG hydrolysate, without and with pH control, and with BY addition, are shown in Table 4. Control of pH increased LA yield by 21%. Lactic acid yield was high in all fermentations with BY addition (84.7% at 5 g/L to 89% at 50 g/L of BY). Yeast addition increased LA yield significantly (by 3.2% at 5 g/L to 8.4% at 50 g/L of BY) compared with the yield obtained in fermentation without BY addition and with pH control. The highest volumetric productivity was achieved after 12 h in all fermentations (Table 4). Much lower volumetric

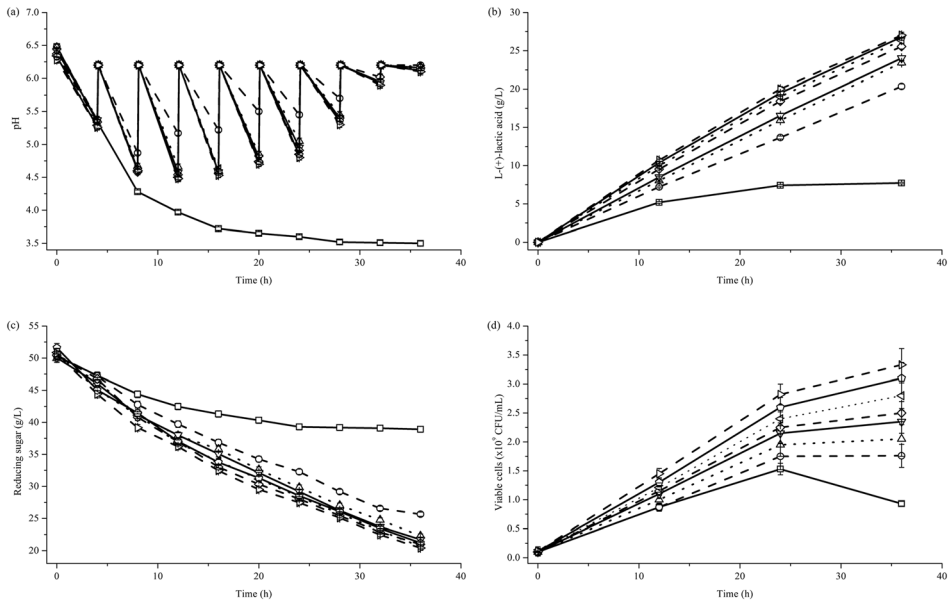

Figure 2. Lactic acid (LA) fermentation of brewer's spent grain (BSG) hydrolysate with various brewer's yeast (BY) content: (a) pH; (b) L-(+)-LA concentration; (c) reducing sugar concentration; (d) *L. rhamnosus* cell viability. Symbols: (□) solid line – without pH control; (○) dashed line – with pH control; (Δ) dotted line – 5 g/L of BY; (▽) solid line – 10 g/L of BY; (◇) dashed line – 20 g/L of BY; (◁) dotted line – 30 g/L of BY; (⌂) solid line – 40 g/L of BY; (▷) dashed line – 50 g/L of BY.

Table 4. Lactic acid [L-(+)-LA] yield and volumetric productivity in L-(+)-LA fermentations of BSG hydrolysate[a]

	L-(+)-LA yield (%)[b]	Volumetric productivity (g/L h^{-1})[c]
Without pH control	67.9 ± 1.3[a]	0.43 ± 0.01[a]
With pH control	82.1 ± 1.5[b]	0.60 ± 0.01[b]
5 g/L brewer's yeast	84.7 ± 1.2[c]	0.67 ± 0.01[c]
10 g/L brewer's yeast	84.8 ± 1.2[c]	0.71 ± 0.01[d]
20 g/L brewer's yeast	86.8 ± 1.1[cd]	0.79 ± 0.01[e]
30 g/L brewer's yeast	87.3 ± 1.3[d]	0.83 ± 0.01[f]
40 g/L brewer's yeast	88.2 ± 1.3[d]	0.87 ± 0.01[g]
50 g/L brewer's yeast	89.0 ± 1.3[d]	0.89 ± 0.01[h]

[a]Values represent means ± standard deviation calculated from three parallel tests. Means with different capital letters in a column are significantly different ($p < 0.05$).
[b]L-(+)-LA yield was calculated at 36 h of the fermentation.
[c]Volumetric productivity was calculated after 12 h of fermentation.

productivity was achieved in fermentations without pH control. The volumetric productivity was significantly higher when BY was added compared with the fermentations without BY addition. Volumetric productivity increased with an increase in BY content from 11.7% at 5 g/L to 48.3% to 50 g/L of BY. The highest volumetric productivity was achieved in fermentation with the addition of 50 g/L of BY (0.89 g/L/h).

BY addition increased the yield and volumetric productivity of lactic acid as it contains proteins, amino acids, minerals, etc. Most *Lactobacillus* require an exogenous nitrogen source of amino acids or peptides to support cell growth *(31)*. *L. rhamnosus* requires a complex nutrient composition for its growth because it lacks an enzyme to synthesise B vitamins and amino acids *(32)*. Hydrolysates obtained from BSG *(27)* and other cellulosic materials such as wheat straw *(33)*, corn cobs *(34)*, wood *(35)*, cassava bagasse, sugarcane bagasse *(36,37)* and corn stover *(8)* required additional nutrients (yeast extract, corn steep liquor, peptone, salts, etc.) for LA production by *Lactobacillus* strains. The results obtained in this study show that BY addition significantly increased LA yield, volumetric productivity and *L. rhamnosus* cell viability.

Sridee *et al. (38)* investigated the addition of BY (8 and 16 g/L) in bioethanol production from sweet sorghum juice by *Saccharomyces cerevisiae* NP 01. The addition of BY (both 8 and 16 g/L) increased bioethanol yield by 2%. A similar increase in bioethanol yield (by 3%) was obtained by Suwanapong *et al. (1)* in bioethanol fermentation of sweet sorghum juice with the addition of BY (21 g/L).

Gao *et al. (39)* used yeast spent cells (6 g/L) in lactic acid fermentation on glucose (100 g/L), without and with salt addition by *L. rhamnosus* NBRC 3863. An LA yield of 46.9% was achieved, which was lower than the yield reported here for the BY content of 5 g/L. Rivas *et al. (40)* evaluated possible application of *Debaryomyces hansenii* spent cells from xylitol production in LA production by *L. rhamnosus* CECT-288 in glucose-containing media (100–120 g/L). In the experiment with the addition of 10 g/L of spent cells, an LA yield of 75% was achieved, which was lower than the yield obtained in this study for the BY content of 10 g/L in hydrolysate. Altaf *et al. (17)* investigated the use of red lentil flour and baker's yeast cells as substitutes for commercial peptone and yeast extract in modified MRS broth medium in LA fermentation by *Lactobacillus amylophilus* GV6. With 20 g/L of red lentil flour and 10 g/L of baker's yeast cells, an LA yield of 92% was achieved, which was higher than that found here. Altaf *et al. (17)* probably obtained a higher LA yield because they replaced peptone and yeast extract in MRS medium with red lentil flour and baker's yeast cells but the medium contained other nutrients and minerals which are found in MRS medium. However, use of the MRS medium could be considered prohibitively expensive.

The price of yeast extract varies and is typically in range of 50–350 $/kg, whereas the price of BY used here was $6 per kg. In our previous study *(19)* the same LA yield of 89% was achieved with the addition of 20 g/L yeast extract, which is comparable with 50 g/L of BY used in this study. In terms of cost, it can be estimated an additional $1 for yeast extract and $0.30 for BY. Also, the addition of 50 g/L BY in BSG hydrolysate increased the reducing sugar concentration by 19 g/L. Taken these considerations into account, the price of yeast extract and glucose in our previous study was $1.1, while the price of BY was $0.3, which represents a 72% reduction cost saving for the nitrogen and carbon source. Accordingly, a partial or complete replacement of yeast extract in LA fermentations could significantly lower the cost of fermentation medium for LA production. A high content of FAN from primarily BY contributed to the higher values of all significant fermentation parameters.

Conclusions

Brewer's yeast was used as a source of nitrogen in LA fermentation where spent grain hydrolysate was used as the main substrate. BY addition significantly increased FAN concentration (by 25% at 5 g/L to 616% at 50 g/L of BY). A positive correlation between FAN concentration in the hydrolysate and LA concentration was found (correlation coefficient 0.913). A high viability of *L. rhamnosus* ATCC 7469 (1.95–3.32 × 10^9 log CFU/mL) was found at the end of all fermentations with BY addition. BY addition increased LA yield and volumetric productivity up to 8.4 and 48.3%, respectively. The highest LA yield (89%) and volumetric productivity (0.89 g/L h^{-1}) were achieved in the fermentation of hydrolysate with 50 g/L of BY.

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